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Nuclear Magnetic Resonance

The combination of NMR spectroscopy, recombinant DNA techniques and theoretical/semiempirical model building appears to be a powerful approach to the protein engineering of antibody combining sites, for the ultimate production of antibodies having desired specificities and functions. In the past year we have sequenced the cDNA's of 12 monoclonal anti spin label antibodies, have constructed a working model of one of these antibodies (M. Levitt), and have carried out a number of NMR experiments that have tested this model and have provided kinetic data on hapten-antibody binding. In the next year crucial tests of this model, our NMR assignments and this general approach to protein engineering will be made by transfection/expression experiments involving specific mutations of the antibody genes for the antibodies AN01-AN12. Keywords:

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PROGRESS REPORT ON CONTRACT N00014-86-K-0388

PRINCIPAL INVESTIGATOR: Harden M. McConnell

CONTRACTOR: Stanford University

CONTRACT TITLE: Determination of the Structural Basis of Antibody Diversity Using NMR

START DATE: 15 June 1986

RESEARCH OBJECTIVE: To employ NMR and recombinant DNA methods to determine and modify the structures of antibody combining sites.

PROGRESS (Year 2)

(1) The cDNA sequences of 12 monoclonal anti DNP-spin label antibodies of the IgG class have been determined, and published.

(2) It has been discovered that two of these monoclonal antibodies (closely related to one another) also bind the co-factor FMN, and this binding competes with the binding of the spin-labeled hapten.

(3) A theoretical model of one of the monoclonal antibodies, ANO2, has been constructed by M. Levitt. The hapten combining site in this model has been identified, and compared with the NMR data. The following NMR data support this model of the hapten combining site.

(a) The combining site has Tryp. 91 in the light chain (Tryp 91L) in stacking interaction with the dinitrophenyl ring of the hapten. NMT (nuclear magnetization transfer) signals between light chain tryptophan protons and a proton of the hapten are observed consistent with this model (#320).

(b) The model provides a natural explanation for the large line width for tyrosine signal G on the light chain; Tyr. 34L is in contact with the hapten and stops rotating when the hapten binds. Evidence for this assignment of the Tyr 43L signal is based in part on the fact that this signal is not seen in ANO1 and ANO3 where Tyr. 34L is replaced by Phe 34L in both cases. A direct NMT between the proton signal attributed to Tyr 34L and a proton of the hapten has been observed (to be published) Asn 35A-H and Gln 89L have also been assigned as being in the binding site on the basis of the model alone. The model also makes understandable some antibody intramolecular NMT signals. See attached figures.

(3) It has been discovered that ANO2 is a weak cryoglobulin; at low temperatures (~ 10°C) the dimerization of Fab fragments can be measured.

(4) The NMR spectra permit an accurate measurement of the kinetics of hapten-antibody binding. At temperatures slightly above room temperature, the on-rate constant is $\sim 10^9$ ℓ/mole, and the activation energy is

5 kcal/mole. This is a classic case of a diffusion limited reaction, where the activation energy reflects the temperature dependence of the viscosity of water. The activation energy for dissociation is 18kcal/mol. See attached figures.

(5) The germ-line gene for the ANO2 light-chain has been isolated and sequenced. There are only two (somatic) mutations in going from the germ line light chain to ANO2 light chain. The heavy chain germ line of ANO7 has been sequenced, and since the light chain is a λ chain (for which there are only four germ lines) we have the germ line genes for the whole antibody.

(6) A short note on the x-ray diffractions of crystals of the ANO2 Fab fragment is in press in J. Mol. Biol. Bob Fox is continuing x-ray work on this structure at Yale, and Dan Leahy is also doing some x-ray work in Huber's lab in Martiensreid.

(7) A substantial number of mutated ANO2 DNA's have been prepared, and attempts made at transfection and expression. Thus far we believe we have been simply defeated in a numbers game - only a small fraction of the transfected cells carry the light chain gene, only a small fraction carry the heavy chain gene, a smaller fraction carry both, and still a smaller fraction expresses both in reasonable amounts.

We are now collaborating with a former graduate student (Tom Frey) at Becton-Dickinson Co., where they have developed methods for such transfections. The ANO2 DNA has now been transfected and expressed in a cell line, and my graduate students are now preparing the mutated sequences to be inserted into the Becton-Dickinson vectors.

Work Plan (Year 3). In year 3 we should be able to test a basic conjecture on which all our work is based: A combination of NMR, theoretical modeling, and recombinant DNA methods provides a practical means for carrying out "protein engineering" of antibody molecules. A critical test will be made when we obtain the NMR spectra of selected ANO2 mutants, especially mutants such as Tyr 34L → Phe 34L. There are half a dozen other mutants under construction, for which NMR spectra will provide a test of the model. All these experiments will test the NMR assignments, and thus test the joint accuracy of the model and assignments.

Assuming the above test is successful, we will move on to the stage of (a) constructing antibody combining sites with novel equilibrium and kinetic properties, and (b) a consideration of the possible ligands of the germ line antibodies.

INVENTIONS (Year 1) None

PUBLICATIONS AND REPORTS (Year 1):

290. "Distances of tyrosine residues from a spin-label hapten in the combining site of a specific monoclonal antibody," Jacob Anglister, Tom Frey and Harden M. McConnell, Biochem. 23, 5372-5375 (1984).

295. "Nonaromatic amino acids inthe combining site region of a monoclonal anti-spin-label antibody," Tom Frey, Jacob Anglister and Harden M. McConnell, Biochemistry 23, 6470-6473 (1984).

298. "NMR technique for assessing contributions of heavy and light chains to an antibody combining site," Jacob Anglister, Tom Frey and Harden M. McConnell, Nature 315, 65-67 (1985).

305. "Diversity of molecular recognition: The combining sites of monoclonl anti spin label antibodies," Harden M. McConnell et al. NMR in the Life Sciences, Plenum Publishing Corp., New York (1986) pp. 87-91.

315. "Translational molecular diffusion in phospholipid monolayers: substrate coupling and phase transitions," M. Seul and H.M. McConnell, J. de Physique 47, 1587-1604 (1986).

317. "Physical chemistry and biological stragety of antigen recognition," H.M. McConnell, ESBA Workshop, Saltsjobaden, Sweden, July 1986. In Structure, Dynamics and Function of Biomolecules, Springer Verlag.

320. "The contribution of tryptophan residues to the combining site of a monoclonal anti DNP anti spin label antibody," Jacob Anglister, Martha Bond, Tom Frey, Daniel Leahy, Michael Levitt, Jim Tomasello, H.M. McConnell and Mei Whittaker, Biocheml 26, 6058-6064 (1987).

329. "Lineshape analysis of NMR difference spectra of an anti-spin-label antibody," Tom Frey, Jacob Anglister and Harden M. McConnell, Biochem. 27, 5161-5165 (1988).

335. "Sequences of twelve monoclonal anti-spin-label antibodies for NMR studies," D.J. Leahy, G.S. Rule, M. Whittaker and H.M. McConnell. Proc. Natl. Acad. Sci. USA Proc. Natl. Acad. Sci. USA 85, 3661-3665 (1988).

TRAINING ACTIVITIES:

Tom Frey received his Ph.D. degree in September 1987. The title of his dissertation is "Magnetic Resonance Studies of a Monoclonal Anti Spin Label Antibody. He is presently doing postdoctoral research for Becton-Dickinson in Mountain View, California.

Daniel Leahy received his Ph.D. degree in March 1988. The title of his dissertation is "Construction of a System to Study the Antibody Combining Site by NMR". He is presently doing postdoctoral research in Munich.

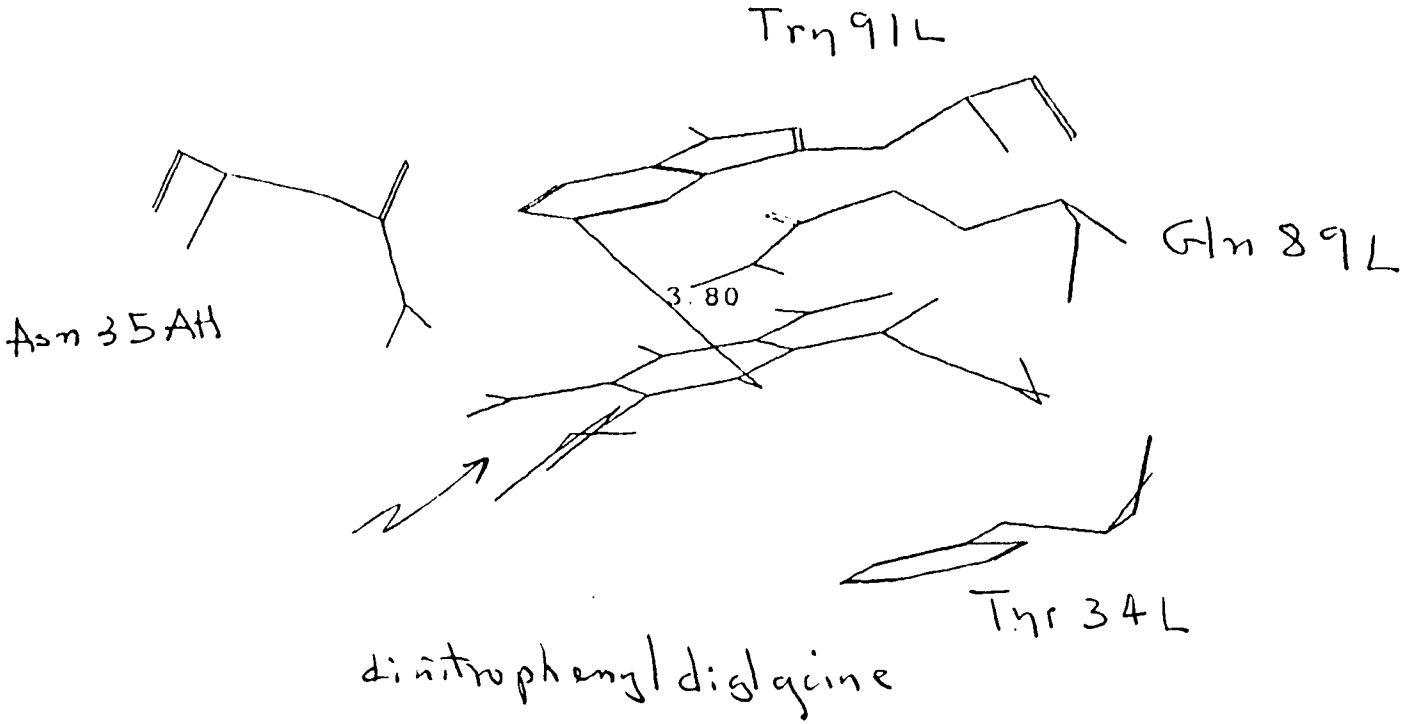
Gordon S. Rule completed two years of postdoctoral research in this laboratory, and is now Professor of Biochemistry, University of Virginia, Charlottesville, Va.

Three graduate students are currently working on this research: Tom Theriault, Barbara Simes-Hooper, Maria Martinez and Pratap Malik.

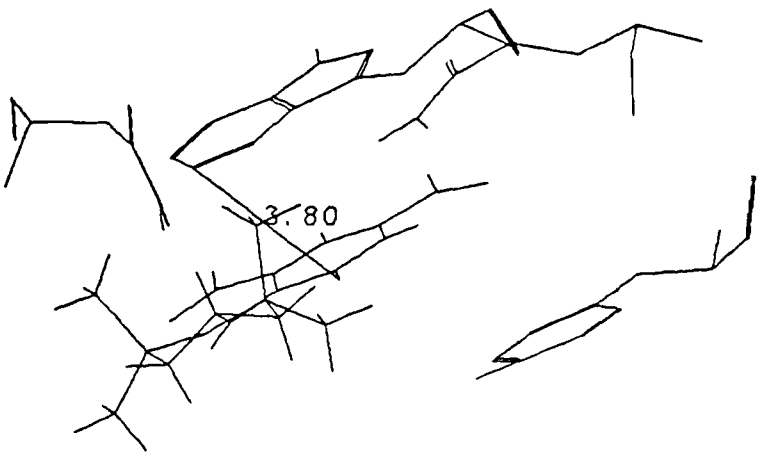
Women/minorities - 2
Non-citizen - 1 (India)

AWARDS/FELLOWSHIPS:

1987-Pauling Medal, Puget Sound and Oregon ACS Sections
1988-Wheland Medal, University of Chicago
1988-U.S. National Academy of Sciences Award in Chemical Sciences



dinitrophenyl diglycine



spin label
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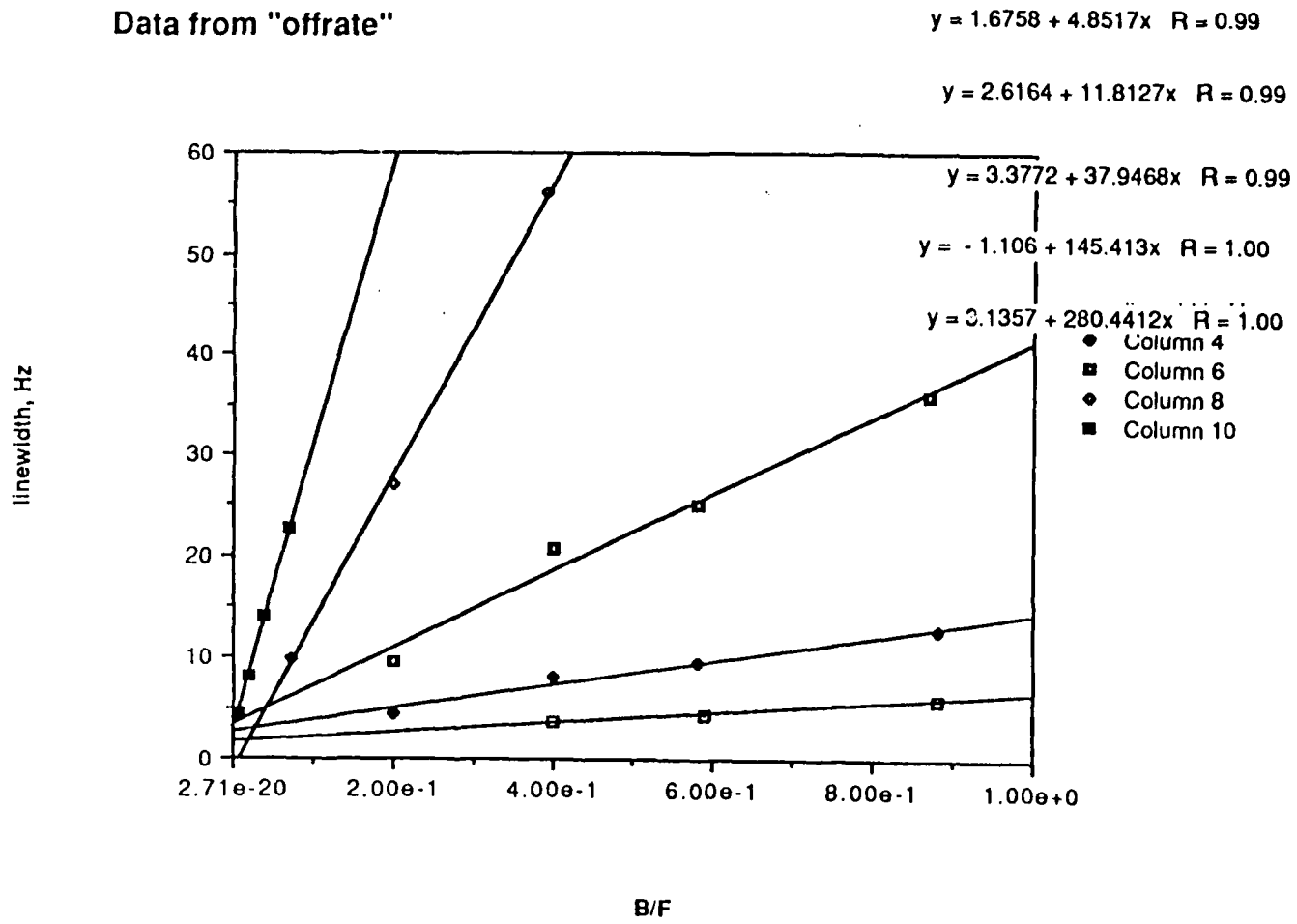

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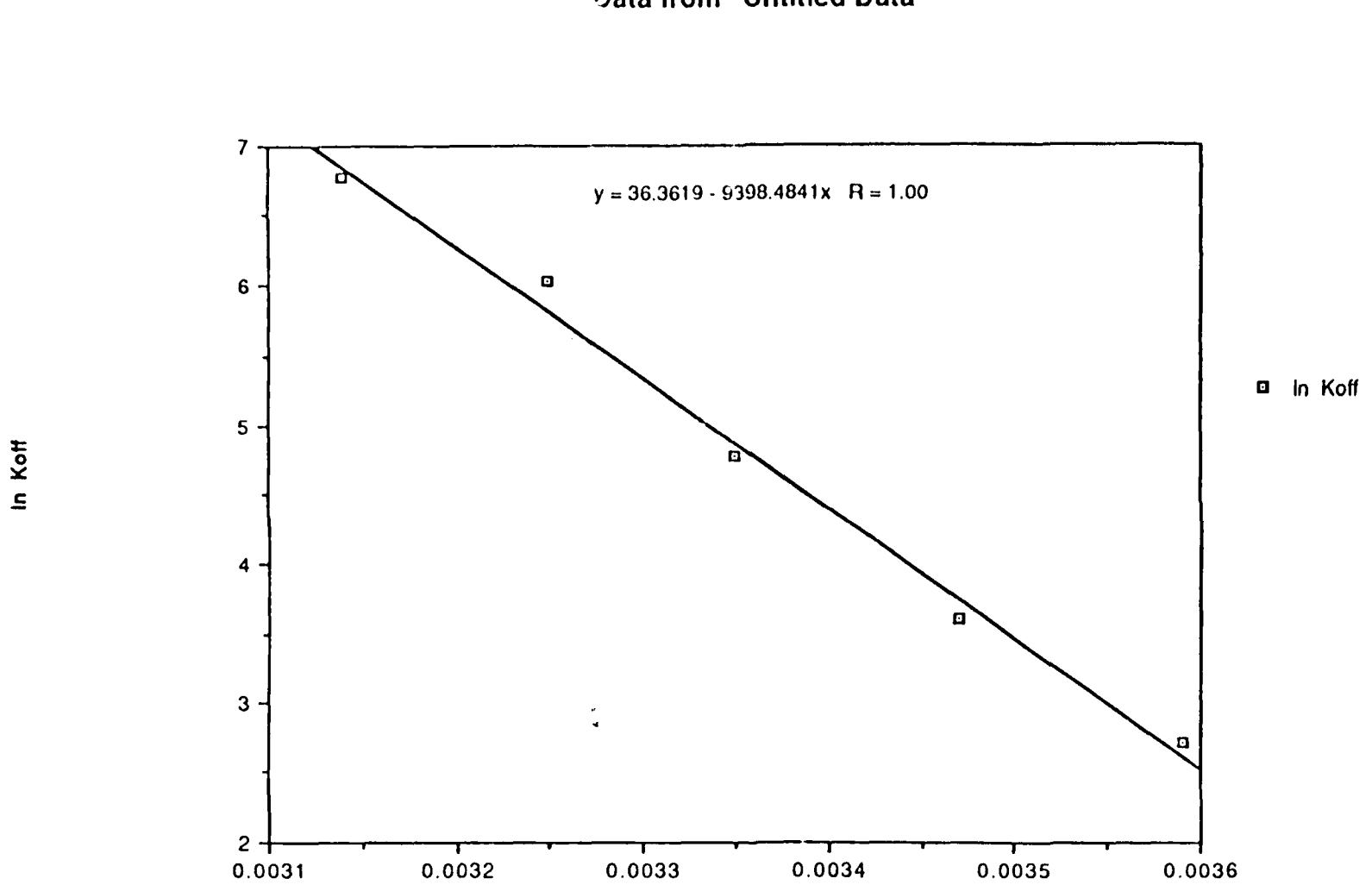

Data for hapten - AN02 "off kinetics"

Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3661–3665, June 1988
Chemistry

Sequences of 12 monoclonal anti-dinitrophenyl spin-label antibodies for NMR studies

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Contributed by Harden M. McConnell, January 19, 1988

ABSTRACT **Eleven monoclonal antibodies specific for a spin-labeled dinitrophenyl hapten (DNP-SL) have been produced for use in NMR studies. They have been named AN01 and AN03–AN12. The stability constants for the association of these antibodies with DNP-SL and related haptens were measured by fluorescence quenching and ranged from 5.0×10^4 M^{-1} to $>1.0 \times 10^8$ M^{-1}. cDNA clones coding for the heavy and light chains of each antibody and of an additional anti-DNP-SL monoclonal antibody, AN02, have been isolated. The nucleic acid sequence of the 5′ end of each clone has been determined, and the amino acid sequence of the variable regions of each antibody has been deduced from the cDNA sequence. The sequences are relatively heterogeneous, but both the heavy and the light chains of AN01 and AN03 are derived from the same variable-region gene families as those of the AN02 antibody. AN07 has a heavy chain that is related to that of AN02, and AN09 has a related light chain. AN05 and AN06 are unrelated to AN02 but share virtually identical heavy and light chains. Preliminary NMR difference spectra comparing related antibodies show that sequence-specific assignment of resonances is possible. Such spectra also provide a measure of structural relatedness.**

Many different magnetic resonance techniques can be used to gain structural information about antibodies in solution (1). The combining sites of antibodies specific for spin-labeled molecules are particularly accessible to study by nuclear magnetic resonance (NMR). The spin-label broadens the NMR signals of nearby (<17 Å) protons in a strongly distance-dependent manner. A simple NMR difference spectrum, antibody alone minus antibody with bound spin-label, is dominated by resonances from protons that are near the electron spin. Anglister *et al.* (2) used this effect to gain information about the amino acid composition of the binding site of AN02, a monoclonal antibody raised against a spin-labeled dinitrophenyl hapten (DNP-SL). Growth of the AN02-producing cell line in medium containing selected deuterated amino acids results in virtually complete incorporation of these deuterated amino acids into the antibody. This selective deuteration permits the assignment of resonances to specific amino acid types. Considerable simplification of spectra is afforded through the use of partially deuterated amino acids to remove splitting of the resonances. Measurement of the broadening effect of the spin-label on specific resonance signals at various binding-site occupancies allows the calculation of the distance between these protons and the electron spin. A complication in this type of distance measurement arises if the spin-label adopts more than one conformation relative to the protein (3). An electron paramagnetic resonance spectrum of AN02 with the spin-label hapten shows the spin-label to be tumbling at the same rate as would be expected for AN02 (2). The distances from the spin-label of seven AN02 tyrosines were measured by varying the amount of spin-label in the binding site (4). Such distance measurements were also made for resonances arising from alanine, isoleucine, leucine, threonine, and valine (G.S.R., D.J.L., and H.M.M., unpublished data). Recombination of heavy and light chains with different deuterations has allowed identification of the chain of origin of these tyrosine signals (5). Nuclear magnetization transfer measurements were used to identify resonance signals from two tryptophans in AN02 that must be <5 Å from the hapten (6).

The NMR lineshapes provide information about the binding-site dynamics. The linewidths of resonance signals originating from residues on the surface of AN02 indicate that these residues are moving much faster than the entire Fab molecule. The linewidths of peaks corresponding to the tyrosine residues for which distance measurements were made varied from 5 to 15 Hz. The sharpness of signals from binding-site protons greatly simplifies analysis of NMR spectra from a protein as large as an Fab fragment. The change in linewidth of a resonance in the AN02 NMR spectrum upon binding of a diamagnetic hapten was used to measure the off-rate for this hapten (4).

Given the available distance information about amino acids near the spin-label in AN02, sequence-specific assignment of resonance signals will enable construction of a working model of the combining site. We believe that such sequence-specific assignments can be obtained in part by using monoclonal antibodies differing from AN02 by a small number of amino acids. We have thus constructed a panel of 12 monoclonal antibodies that bind the DNP-SL hapten. We have also isolated and sequenced cDNA clones for both chains of each of these antibodies.* These clones enable creation of related antibodies through site-directed mutagenesis, genetic mixing and matching of heavy and light chains, and construction of hybrid variable (V) regions. Preliminary data given below demonstrate NMR difference spectra between closely related antibodies to be a viable technique to obtain sequence-specific assignments.

The Fab fragment of the AN02 antibody forms cocrystals with the DNP-SL hapten that diffract to high resolution (D.J.L., H.M.M., and R. O. Fox, unpublished data). The solution of the AN02 crystal structure will provide an opportunity to compare NMR and x-ray structural information for antibody–hapten complexes.

MATERIALS AND METHODS

Immunization of Mice. Six- to 8-week old female BALB/c mice were given intraperitoneal injections of the DNP-SL hapten coupled to 250 μg of bovine serum albumin (DNP-SL-BSA) in a 1:1 emulsion with complete Freund's adjuvant. The DNP-SL-BSA complex was prepared by the method

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Abbreviations: DNP-SL, spin-labeled dinitrophenyl; *C*, constant; *D*, diversity; *J*, joining; *V*, variable.

*The sequences reported here are being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession nos. J03832 and J03833).

described for DNP-SL-keyhole limpet hemocyanin (7). After 2 weeks the mice were boosted with subcutaneous injections of ~50 μg of DNP-SL-BSA in complete Freund's adjuvant. Two weeks later, sera from these mice were screened by ELISA for anti-DNP-SL activity. Mice with the highest anti-DNP-SL titers were then injected with 70 μg of soluble DNP-SL-BSA in aqueous solution via the tail vein. The spleens were removed for fusion 3 days later.

ELISA. DNP-SL-lipid (7) at 2.5 mg/ml in $CHCl_3$ was diluted 1:100 with ethanol and coated on the wells of a 96-well polystyrene plate by evaporation. The wells were washed five times with 0.3% gelatin in phosphate-buffered saline and flicked dry. Appropriately diluted serum or supernatant was added to the wells and incubated for 1 hr. The plates were washed as before and specific antibody binding was detected with β-galactosidase-conjugated goat anti-mouse immunoglobulin (Bethesda Research Laboratories) followed by an appropriate enzyme substrate.

Preparation of Hybridomas. Exponentially growing Sp2/0 cells (2×10^7) were mixed at a 1:5 ratio with immune spleen cells. The cells were washed three times in serum-free RPMI-1640 medium and gently resuspended in 1 ml of RPMI-1640 containing 40% (wt/vol) polyethylene glycol (PEG; M_r 3500–3700; Baker) and 10% (vol/vol) dimethyl sulfoxide; this solution was added dropwise at 37°C over 1 min. The cells were incubated at 37°C for 2 min and then diluted slowly with 5% PEG in RPMI-1640. The cells were pelleted by centrifugation and resuspended in 5 ml of serum-free RPMI-1640. The suspension was then brought to 100 ml with RPMI-1640 containing 20% (vol/vol) fetal bovine serum (HyClone, Logan, UT), 2 μg of azaserine per ml, and 0.2 mM hypoxanthine, and the cells were seeded in 96-well plates. The cells were fed every 3–4 days, and supernatants were screened by ELISA for anti-DNP-SL activity after the appearance of colonies. Colonies testing positively were immediately cloned and later recloned until all new subclones tested positively. All cell lines were subsequently adapted for growth in medium containing 1% fetal bovine serum to minimize the presence of protonated amino acids during deuteration experiments for NMR.

cDNA Cloning. RNA was isolated from each cell line by the method of Chirgwin *et al.* (8), and poly(A)$^+$ RNA was selected with oligo(dT)-cellulose (Collaborative Research, Waltham, MA). A complementary strand of DNA was synthesized by reverse transcriptase (Life Sciences, St. Petersburg, FL) using an oligo(dT) primer (Pharmacia). The RNA was removed by heating to 90°C and digestion with RNase A (Sigma). The single-stranded cDNA was poly(G)-tailed by terminal deoxynucleotidyltransferase (Pharmacia) using dGTP (Pharmacia). Second-strand synthesis was primed with an oligo(dC) primer that contained a *Not* I restriction site on its 5′ end (D. W. Denney and T. St. John, personal communication). The DNA was then methylated with *Eco*RI methylase and ligated to *Eco*RI linkers (New England Biolabs). The methylated DNA was digested with *Eco*RI, size-selected by electrophoresis in an agarose gel, and ligated into *Eco*RI-treated λgt10. The resulting phage library was screened with hybridization probes that had been labeled with [α-^{32}P]dCTP (New England Nuclear) by the random hexamer method (9). The probes consisted of DNA encoding the constant (*C*) region of γ heavy chain or κ or λ light chain. Positive phage were purified, and the cDNA inserts were sized by electrophoresis and then were subcloned as *Not* I–*Eco*RI fragments into pUC18N, mp18N, and mp19N. These are standard pUC and M13 mp vectors that have been modified to contain a *Not* I site in the polylinker.

DNA Sequencing. All clones were sequenced by the dideoxy method with 2′-deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate (New England Nuclear) as label (10, 11). Two 60-cm gradient gels, one run for 30,000 V·hr and one run for 8500 V·hr, were sufficient to sequence the entire *V* region with the use of a single primer. The antisense strands were sequenced by using the standard M13 primers, and the coding strands were sequenced with the following primers, which are complementary to regions on the 5′ end of the appropriate constant region: 5′ d(CAGGGTCACCATGGA) 3′ (γ), 5′ d(CACGACTGAGGCACC) 3′ (κ), and 5′ d(GCTCCTCAGAGGAGGGC) 3′ (λ). Multiple independent clones were sequenced for >75% of the chains.

Preparation of DNP-SL. The synthesis of the spin-labeled hapten has been described (7). The chemical formula can also be found in Anglister *et al.* (2).

NMR Spectra. Samples were prepared for NMR as described (2). NMR spectra were obtained in Fourier-transform mode on a General Electric NMR spectrometer operating at 500 MHz (proton). Approximately 6000 scans of 16,000 data points with a sweep width of ±4000 Hz were taken for each spectrum. Residual water protons were presaturated, and chemical shifts are given relative to a tetramethylsilane standard.

RESULTS

Eleven new anti-DNP-SL monoclonal antibody-producing cell lines were generated from the spleens of five mice. The isotypes, subgrouping according to Kabat *et al.* (12), joining (*J*)-region usage, and stability constants of these antibodies are shown in Table 1. All antibodies are of the IgG class and have stability constants for DNP-SL ranging from 5.0×10^4 M^{-1} to 7.3×10^7 M^{-1}. The previously described (2) monoclonal anti-spin-label antibody was given the name AN02, so we have named these 11 new antibodies AN01 and AN03–AN12. Balakrishnan *et al.* (7) referred to an antibody named AN01, but it later proved identical to AN02. We have thus used the name AN01 for one of the new antibodies.

The deduced amino acid sequences of the heavy- and light-chain *V* regions of each antibody are shown in Figs. 1 and 2, respectively. The nucleic acid sequences are being contributed to the GenBank data base* (13), and copies are available upon request. This panel of anti-DNP-SL antibodies is not dominated by sequences originating from one *V*-region gene family. There are subgroups, however, that do show a high degree of similarity among themselves. Fig. 3 indicates which heavy and light chains have highly similar DNA sequences. Both the heavy and the light chains of AN01 and AN03 utilize *V* regions derived from the same families as those of AN02. The heavy chain of AN07 and the light chain of AN09 are similarly related to AN02. These heavy and light chains show no preferred diversity (*D*)- or *J*-region usage. All gene segments of the AN05 and AN06 antibodies are similar if not identical in origin.

In addition to clones coding for the expressed antibody proteins, cDNA clones coding for an aberrant γ or an aberrant κ chain were found. The aberrant γ clones were always identical in sequence, as were the aberrant κ clones, although they were found in several libraries constructed from independent hybridoma cell lines. The κ-chain sequence was identical to the sequence found and characterized in several fusion partners of common lineage with Sp2/0 (S. Levy, personal communication). The aberrant γ-chain sequence consisted of a portion of the intron between the *J* and *C* coding regions spliced to the $C_\gamma 1$ exon. Examination of the *J*–*C* intron sequence listed in the GenBank data base† (13) revealed a splice donor site, 5′ d(AAATG/GTAAGCC) 3′ (14), at sequence positions 4189–4200 that would generate the observed sequence. The 5′ end of each of these clones

*EMBL GenBank Genetic Sequence Database (1987) GenBank (IntelliGenetics, Mountain View, CA), Tape Release 54.

Table 1. Characteristics of the monoclonal antibodies

Name	KH	KL	H	L	J_H	J_L	Stability constant, M^{-1} DNP-SL	DNP-SG	DNP-G_2	DNP-SA	DNP-D_2	DNP-en_2	DNP-G
AN01	Ia	VI	γ1	κ	J_H2	$J_κ4$	5.1×10^4						
AN02	Ia	VI	γ1	κ	J_H3	$J_κ5$	7.5×10^6	1.8×10^7	3.1×10^6	2.0×10^7	2.7×10^5	6.0×10^6	4.5×10^6
AN03	Ia	VI	γ1	κ	J_H2	$J_κ5$	6.2×10^4	Low	Low				
AN04	IIa	V	γ1	κ	J_H3	$J_κ1$	6.8×10^6	4.4×10^7	2.2×10^6	2.9×10^6	Low		
AN05	IIc	II	γ1	κ	J_H2	$J_κ5$	9.5×10^6	9.2×10^6	Low	1.2×10^7			
AN06	IIc	II	γ1	κ	J_H2	$J_κ5$	1.2×10^7	2.7×10^7	Low	2.6×10^7			
AN07	Ia	I	γ1	λ	J_H3	$J_λ1$	2.5×10^7	$>5 \times 10^7$	4.9×10^5	9.7×10^5	Low		
AN08	IIa	V	γ1	κ	J_H4	$J_κ2$	7.3×10^7	3.0×10^7	1.7×10^6	2.4×10^6	Low		
AN09	IIa	VI	γ2a	κ	J_H3	$J_κ5$	1.6×10^7	$>1 \times 10^8$	4.0×10^7	$>1 \times 10^8$	2.7×10^6		
AN10	III	I	γ1	λ	J_H4	$J_λ1$	4.9×10^7						
AN11	IIa	V	γ1	κ	J_H1	$J_κ2$	5.0×10^5						
AN12	IIa	V	γ1	κ	J_H3	$J_κ2$	2.1×10^6						

Subgrouping [according to Kabat *et al.* (12)] for the heavy (KH) and light (KL) chain, isotype for the heavy (H) and light (L) chain, *J*-region usage for the heavy (J_H) and light (J_L) chain, and stability constant for various DNP haptens are shown for each antibody. The haptens were as follows: DNP-TEMPO-ethylenediamine (DNP-SL), DNP-TEMPO-glycine (DNP-SG), DNP-bis(glycine) (DNP-G_2), DNP-TEMPO-aspartic acid (DNP-SA), DNP-bis(aspartic acid) (DNP-D_2), DNP-bis(ethylenediamine) (DNP-en_2), DNP-glycine (DNP-G). TEMPO is 2,2,6,6-tetramethyl-1-oxypiperidin-4-yl. All haptens were synthesized by amine linkage of the substituents to the 2 and 4 positions of 2,4-difluoro-1,5-dinitrobenzene except DNP-glycine, which was purchased from Sigma.

extended to the *Eco*RI site at positions 3559–3564 of the *J–C* intron. This termination site is probably an artifact of our size selection and inadequate methylation during construction of the libraries. These transcripts are probably nonfunctional, as they contain several in-frame stop codons. The presence of this clone in a library expressing a γ2a heavy chain indicates that it is not solely due to missplicing of the expressed transcript. The aberrant γ sequence constituted ≈10% of γ clones screened from all libraries.

Sequencing of multiple clones revealed occasional differences between clones encoding the same polypeptide chain. Of ≈25,000 overlapping sequence measurements, 4 single-base-pair substitutions were detected. The errors appeared to be random and indicated a small but nonnegligible error rate (on the order of 1 error per 6000 base pairs) in our cloning procedures.

A 7-base-pair insertion was observed in one of the clones coding for the AN04 κ chain. The correct sequence at the insertion site, as determined from two independent clones, consisted of a contiguous repeat of the 7-base-pair sequence 5′ d(CAGCCTG) 3′. In the abnormal clone this sequence was repeated three times, suggesting a slippage or "hiccup" of the polymerase during chain elongation. Error due to the slipping of a polymerase between direct repeats has been postulated as a mechanism of somatic mutation in immunoglobulins (15).

A series of abnormalities was also observed in the leader sequence of AN02 heavy chain clones near the initiating

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                -15       -5    1        10        20        30   35 36
                 |         |    |        |         |         |    AB
AN02     MRVLILLWLFTAFPGILSDVQLQESGPGLVKPSQSQSLTCTVTGYSITSDYAWN WI
AN01     .K..S..Y.L..I.....................L....S.......G.Y.. ..
AN03     .K..S..Y.L..I.....................L....S.......G.Y.. ..
AN07     .......C.................D.......L.............G.S.H ..
AN05     ME.HW.F.F..SVTA.VH.QF.P.Q..AE.A..GA.VKMS.KAS..TF..YWMH   .V
AN06     ME.HW.F.F..SVTA.VH.Q..P.Q..AE.A..GA.VKMS.KAS...F.RYWMH   .V
AN04     MGWSW.F.F.LSGTA.VHCQI..KQ...E....GA.VKIS.KAS...F.DY.IN   .V
AN09     MGWSY.I.F.VATATDVH.Q....QP.AE....GA.VK.S.KAS..TF..YWMH   .V
AN11       MSW.F.F.LSGTA.VH.E....Q...E..R.GA.VKMS.KAS..TF..YWMH   .V
AN12     MEWNWVV.F.LSLTA.VYAQG.M.Q..AE....GA.VK.S.KTS.FTFR.S.IG   .L
AN08     MEWLWN..F.MA.AQS.QAQI..VQ...E.K..GETVRIS.KAS..TF.TAGIQ   .V
AN10     MNFGFS.IF.VLVLK.VQCE.K.V...G.....GG.LK.S.AAS.FTFS.YAMS   .V

                40          52  53      60          70          82  83      90
                 |           ABC  |       |           |           ABC  |       |
AN02     RQFPGNKLEWMGYMS    YSGSTRYNPSLRSRISITRDTSKNQFFLQLKSVTTEDTATYF
AN01     ..............IN    .D.RNN.....KN..............K.............Y
AN03     ..............IN    .D.NNN.....KN..............K.N...........Y
AN07     .....H.......IH     .....N.....K..................N...........Y
AN05     K.R..QG...I..INP    NT.Y.V..QKFKDKATL.A.K.SSTAYM..S.L.SD.S.V.Y
AN06     K.R..QG...I..INP    ST.Y.E..QKFKDKATL.A.K.SSTAYM..S.L.S..S.V.Y
AN04     K.K..QG...I.WIYP    G...NNK..EKFK KATL.I...SSTVYI..S.L.S....V..
AN09     ..R..CG...I.EINP    SN.R.N..EKFK.KATLNV.K.SSTAYM.IS.L.S..S.V.Y
AN11     K.K..QG...I..INP    .NDG.K..EKFKGKATL.S.K.SSTAYIE.S.L.S..S.V.Y
AN12     K.K..QS...IAWIYA    GT.G.S..QKFTGKARL.V...SSTAYM.FS.L....S.I.Y
AN08     QKM..KG.K.I.WINT    R...VPK.AEDFKG.FAFSLE..ASTAY..ISNLRND...A..
AN10     ..T.ERR...VASI.     SGYI.Y.PD.VKG.FT.S..NAR.ILY..MS.LRS....M.Y

                95   100              105  110
                 |    ABCDEFGHIJK     |    |
AN02     CARGWP              LAYWGQGTQVSVSE
AN01     ...EDDGYYI           FD.....STLT..S
AN03     ...EGYGYF            FD.....TLT..S
AN07     ...VIYYYGSSYV       WF.....L.T..A
AN05     ..YYGSS             YFD.....TLT..S
AN06     ..HYGRS             YFD.....TLT..S
AN04     .V.YGYDG             FG.....L.T..A
AN09     ...R.GSYVGG          F.....NM.T..A
AN11     ...FGYYGR          YWYFDV..A..T.T..S
AN12     ...WD.INRG           F.....L.T..A
AN08     .G.TDYYGST         YYAMD.....SS.T..S
AN10     ...WGHRYDVL          D.....S.T..S
```

FIG. 1. Deduced amino acid sequences of the *V* regions of the heavy chains of the anti-DNP-SL monoclonal antibodies AN01–AN12. Numbering system is according to Kabat *et al.* (12). Standard one-letter amino acid symbols are used.

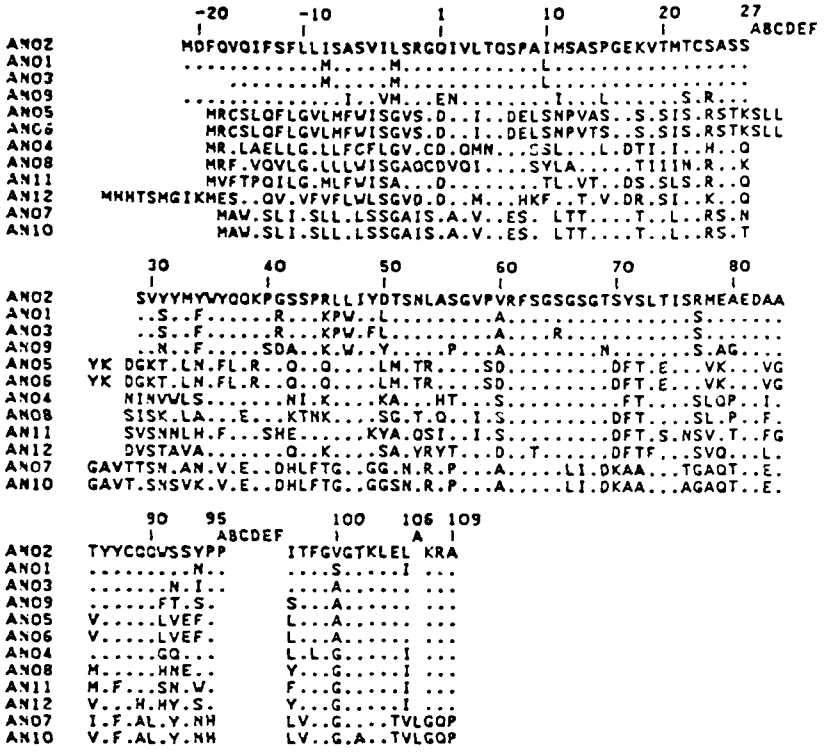

FIG. 2. Deduced amino acid sequences of the *V* regions of the light chains of the anti-DNP-SL monoclonal antibodies AN01–AN12. Numbering system is according to Kabat *et al.* (12).

ATG. Of four independent cDNA clones sequenced, three different sequences were found in this region (Fig. 4), one of which was identical to the sequence of this region from a genomic clone of the AN02 heavy chain. Plasmids containing the non-germ-line versions of this gene appear to lyse the host bacteria before saturating growth conditions are reached.

DISCUSSION

In Fig. 5, spectra a and b show the aromatic regions in an NMR difference spectrum, unoccupied Fab minus Fab occupied with a low concentration of DNP-SL, for AN05 and AN06, respectively. The signals in these spectra originate from protons in close proximity to the spin-label (<10 Å). There is only one sequence difference between AN05 and AN06 variable regions that involves aromatic residues. AN05 has a tyrosine at position 94 of the heavy chain, whereas AN06 has a histidine at this position. Spectrum c is the double difference spectrum obtained by subtracting spectrum b from spectrum a. This spectrum clearly reveals four negative signals, which are likely to arise from the tyrosine-94 protons of AN05, and two positive signals, which are candidates to arise from the histidine-94 protons of AN06. The upfield chemical shift of the positive features is unusual for histidine residues but not without precedent (16). Further experiments, such as selective deuteration, are necessary before a detailed interpretation of these spectra can be made.

The simplicity of the double difference spectrum, c, in Fig. 5 shows that the binding sites of AN05 and AN06 have nearly identical conformations. This result illustrates how simple NMR difference spectra can provide a useful measure of the structural relatedness between antibodies of highly similar sequence. This approach may also be of use for other families of closely related proteins for which a limited number of structures are known.

Both AN05 and AN06 have a yellow color after elution from a protein A-Sepharose column. This coloring could not be removed by extensive dialysis. Preliminary experiments show this color to be due to bound riboflavin, presumably from the culture medium. AN05 and AN06 also bind flavin mononucleotide and flavin adenine dinucleotide. This binding of DNP-SL and these flavins is competitive.

AN01 and AN03 are similar enough in sequence that NMR difference spectra, in combination with deuteration, should yield some sequence-specific assignments. AN01 and AN03

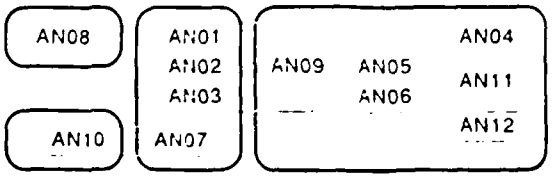

FIG. 3. AN01–AN12 grouped according to those that have closely related chains. Dark lines enclose antibodies whose heavy-chain nucleic acid sequences are >75% identical. Lighter lines enclose antibodies whose light-chain nucleic acid sequences are >85% identical. A different criterion was used for heavy chains because this distinction provided the clearest discrimination between sequences.

	-17	-16	-15	-14	-13	-12
1.	GCC	TAA	AAG	ATG	AGA	GTG	CTG	ATT	CTT
2.	CAG	CCT	CCA	GTG	AGA	GCG	ATG	ATT	CTT
3.	CAG	CCT	CCA	GTG	AGA	GCG	CTG	ATT	CTT

FIG. 4. Nucleic acid sequences of the leader regions of different cDNA clones encoding the AN02 heavy chain. The codons are numbered relative to the last residue of the leader peptide (−1). The sequences 3′ to those shown were identical among all clones and coded for an otherwise normal heavy chain. Sequence 1 is identical to the sequence for this region found in a genomic clone of the AN02 heavy chain. Sequence 2 was found in two independent cDNA libraries constructed from different preparations of RNA. Sequence 3 did not contain an apparent initiating ATG codon. These differing leaders do not arise from an identifiable splicing event (14).

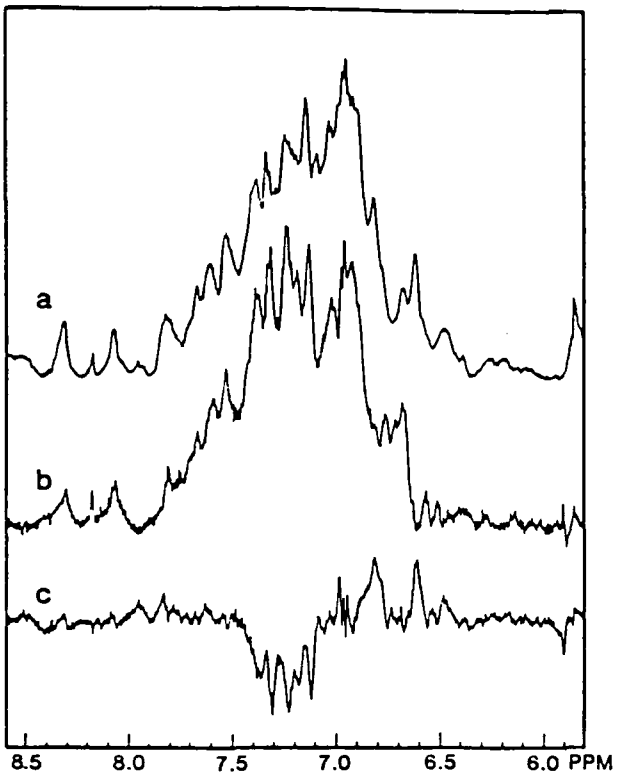

FIG. 5. Aromatic regions of the difference spectra, unoccupied Fab minus Fab with 4% (mol/mol) bound spin-label, for AN06 (spectrum a) and AN05 (spectrum b). Spectrum c is the double difference spectrum a minus b.

differ enough from AN02 that direct comparison of NMR spectra is unlikely to yield sequence-specific assignments for AN02. The number of amino acid differences in the *V* regions and the use of different leader sequences indicate that both chains of AN02 are derived from related but different germ-line genes as those of AN01 and AN03. AN02 has 100-fold higher affinity for DNP-SL than do AN01 and AN03. Site-directed mutagenesis should allow identification of residues important for high-affinity binding in these related antibodies. Cloning and sequencing of the germ-line genes that gave rise to the AN02 *V* regions should enable the construction of mutants for investigation of structural pathways selected during somatic mutation.

The remaining antibodies use unique combinations of heavy and light chains. The diversity found in these anti-DNP-SL antibodies provides a rich source for structural and functional studies. The creation of new antibodies with recombinant techniques further increases the diversity of this system. Mixing and matching heavy and light chains combinatorially increases the number of related antibodies within a subgroup and provides for chain-specific assignments. Restriction endonuclease sites can be used to create hybrid chains. For example, restriction sites in the third framework region of the heavy chain allow switching of the *D-J* combination in cases where similar *V*, but different *D* and *J*, regions are used.

NMR studies provide kinetic information in a time range particularly relevant to hapten binding (H.M.M., T. E. Frey, and J. Anglister, unpublished data). Construction of mutants that affect the on-rates or the off-rates of specific haptens should reveal structural features relating to chemical kinetics (17).

The NMR techniques used to study the combining sites of monoclonal anti-spin-label antibodies can clearly be extended to other proteins with modest affinities for spin-labeled ligands. Modification of ligands with spin-labels, in combination with amino acid deuteration and recombinant DNA techniques, promises to allow detailed analyses of protein binding sites by NMR.

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Line-Shape Analysis of NMR Difference Spectra of an Anti-Spin-Label Antibody†

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ABSTRACT: Specifically deuteriated Fab fragments of the anti-spin-label antibody AN02 were prepared. NMR difference spectra were obtained, in which the spectrum of Fab with some fraction of the binding sites occupied with spin-label hapten was subtracted from the spectrum of Fab with no spin-label. The peak heights were analyzed as a function of the fractional occupation of the binding site, using a computer program that calculates a best fit to the observed spectra. This method treats all of the peaks in the spectra simultaneously. Analyzing all peaks at once allows for the interdependencies in the spectra arising from overlap of positive and negative signals from different peaks. The fitting program calculates line widths for the peaks arising from protons in the binding site region. Almost all of the line widths calculated for the spectrum of the Fab complex with diamagnetic hapten dinitrophenyldiglycine were found to be narrower than the line widths of the corresponding resonances in the spectrum of Fab with an empty binding site. The distances of the binding site region protons from the unpaired electron of the hapten were also obtained from this calculation. Two tyrosine protons were found to be close (<7 Å) to this electron. These line-width and distance results are discussed with respect to the structure and dynamics of the antibody binding site.

In an effort to improve the understanding of antibody binding site structure, we have pursued an NMR study of the anti-spin-label antibody AN02. In previous work, we used biosynthetic deuteriation and protein chemistry with NMR to obtain information about the amino acid makeup of the binding site region (Anglister et al., 1984a; Frey et al., 1984) and to assign resonances to the heavy and light chains (Anglister et al., 1985). Nuclear magnetization transfer was used to identify the resonances of protons in close proximity to the hapten (Anglister et al., 1987). The effect of the paramagnetic hapten on the protein NMR spectrum was used to measure the distance of certain antibody protons from the unpaired electron of the hapten (Anglister et al., 1984b).

The effect of the hapten on the AN02 resonance spectrum was described, and a technique for extracting distance information was discussed. This technique involves titrating the binding site of the Fab with paramagnetic hapten and subtracting the NMR spectrum taken at each titration point from the spectrum of the Fab with no hapten bound. The change in peak height of each resonance was analyzed as a function of fractional occupation of the binding site. Several tyrosine resonances displayed the theoretically expected behavior.

The technique presented in our earlier paper analyzed one signal at a time, required that the line width of the proton resonance be known, and assumed that the resonance signals were homogeneous and Lorentzian. Of course, this is not satisfactory for spectra with multiple, overlapping resonances. Further difficulties arise with line-width determination when resonances are very broad or very narrow, due to low signal to noise and digitization, respectively. In the present work, we find that simulation and computer fitting of the observed spectra can be used successfully to extract the desired line-width and distance information.

Materials and Methods

The preparation and purification of the AN02 antibody have been described. Fab fragments were prepared and purified as previously described. The affinity of AN02 for spin-label

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has been reported to be 4×10^6 M^{-1} (Anglister et al., 1984a).

The Fab fragments used for the spectra in this paper were biosynthetically deuteriated in the tryptophan and phenylalanine residues, and in the 2- and 6-positions of the tyrosine rings. The only proton resonances seen in the spectral region of 6.0–9.0 ppm are therefore from the 3- and 5-protons of tyrosine and possibly from histidine. We have shown that histidine makes at most only a minor contribution to any of these difference spectra (Anglister et al., 1984b).

Three types of difference spectra were obtained. In the first type, the spectrum of Fab with spin-label bound was subtracted from the spectrum of Fab with an empty binding site. In the second type, the spectrum of Fab with spin-label bound was subtracted from Fab with dinitrophenyldiglycine (DNP) bound. In the third, the spectrum of Fab with spin-label bound was subtracted from that of Fab with chemically reduced spin-label bound. (In the reduced spin-label, the NO of the nitroxide group is replaced by the hydroxylamine group, NOH.) The chemical shifts of Fab proton resonances seen in this third type of spectrum were assumed to correspond to the chemical shifts in the Fab spin-label complex.

Two types of difference spectra titrations were recorded. In the "without minus with" titration, one subtracts the NMR spectrum of an Fab fragment in a given solution with spin-label from the spectrum of Fab in the absence of spin-label. Different difference spectra are recorded for different spin-label concentrations, corresponding to different fractional occupations of the combining site. In the "with DNP minus with spin label" titrations, one subtracts the NMR spectrum of an Fab fragment in a solution containing both spin-label *and* DNP-diglycine from the spectrum of a solution of Fab with (excess) DNP-diglycine from the spectrum of a solution of Fab with (excess) DNP-diglycine alone. The relative concentrations of spin-label and DNP-diglycine, together with their known binding constants, determine the fractional occupation of the binding site by each hapten. This relative fractional occupation is varied during the "with DNP minus with spin label" titration. Note that this second titration is distinct from that used earlier (Frey et al., 1984).

NMR spectra were simulated on an IBM-PC. Broadening due to the spin-label was described by the equations for paramagnetic broadening (Sternlicht et al., 1965a,b; Jardetzky & Roberts, 1981), giving an r^{-6} dependence for the effect (Solomon, 1955). Spectra for partially occupied situations were assumed to conform to the Bloch equations for one-proton, two-site exchange with an appropriate exchange rate (Gutowsky et al., 1953; McConnell, 1958). Rates found for these experiments are in a time regime intermediate between fast and slow exchange. Initial estimates for spectral parameters were improved by the Marquardt algorithm using a modification of the CURFIT routine of Bevington (1969). The programs were written in C, with time critical portions recoded in 8088 and 8087 assembly language, based on the assembly language output of the C compiler (Computer Innovations) [for further details, see Frey (1986)]. The spectra were digitized at 1-Hz intervals.

RESULTS

The best fit to the data uses an off rate of 430 s^{-1}. This gives a total squared deviation from the data of about 0.25% of the total squared amplitude. This fit is an order of magnitude better than when all distances are set equal to 10 Å and when the best-fit line widths are used. The value of 0.25% is probably set by the noise in the spectra. A further source of error may be the very low amplitude features that appear in the high-occupancy difference spectra and that are ignored by the fit.

The fitting algorithm used finds the parameters giving a minimum in the sum of the squares of the deviations of the calculated amplitudes from the observed data. The only free parameters in the model are the line width of the signal in the Fab with an unoccupied binding site, the line width of the same resonance in the with DNP complex, and the distance of the proton from the unpaired spin in the with spin-label complex. Thus, there are 3 parameters per proton to be fit in each data set (30 for the data set discussed in this paper). There is one observed amplitude per peak per titration point in the data set (108 points). Note that peak B is a composite of 2 signals (Anglister et al., 1985) and there are therefore 9 peaks and 10 protons. The fit is still overdetermined even for this many parameters, but the derived values for the two signals from peak B are given with less confidence. Further complicating the interpretation of peak B is the possibility of a third signal forming a shoulder on the high chemical shift side in difference spectra with high spin-label occupancy.

Some assumptions are implicit in the analysis of these NMR spectra. The scaling of the spectra assumes that at least one line width in one spectrum can be measured acurately. For the present calculations, the lines labeled H and G in the without minus with 100% spin-label spectrum were used for scaling all of the without minus with spin-label spectra. These special peaks were selected on the basis of the following criteria. (i) The location of the base line is clear. In these difference spectra, the summing of negative signals in regions with many resonances can affect the shape of the base line even when any one of these negative signals is too broad to make a noticeable effect. (ii) There is no overlap with other resonances. (iii) The protons are sufficiently near the unpaired electron that the contribution of the negative signal in the difference spectrum is completely obliterated at 100% occupancy. (Note that when proton signals of different width are subtracted the width at half-height of the resulting feature is not the line width of either proton signal.) (iv) The lines are narrow enough to provide good signal to noise but broad enough to provide enough data points to minimize error from the discontinuous nature of the digital spectrum. The measured line widths should also yield relative peak amplitudes that are consistent with those observed in the spectra. Using similar criteria, we used line H to scale the with DNP minus with with spin-label titration.

A second set of assumptions concerns the spectral position of resonances in the Fab spin-label complex. The chemical shifts of several tyrosine resonance change with the occupation of the binding site. Since these resonances are extremely broad in the spectrum of the Fab spin-label complex, it is not possible to determine the chemical shift directly. These shifts are therefore assumed to be the same as those observed in the complex with reduced spin-label. The chemical shifts of resonances in the reduced spin-label complex are very similar to those in the DNP complex, and so this likely to be a good approximation (Anglister et al., 1985).

Another question arises when treating the with DNP minus with spin-label spectra. The system was analyzed as a two-state system involving only the DNP complex and the spin-label complex. The concentrations of DNP and spin-label were kept high enough to saturate all the binding sites, and the concentration of Fab was high enough to make the concentration of free Fab negligible. The Appendix shows that under these conditions a three-state NMR line-shape problem is reduced to the two-state treatment used.

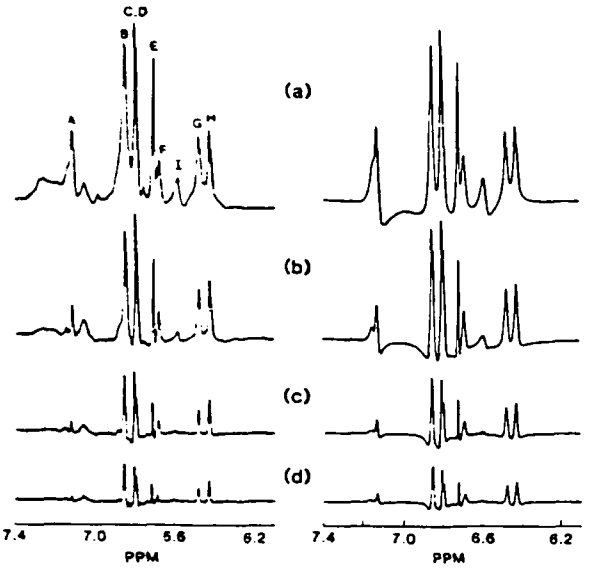

FIGURE 1: Difference spectra of without spin-label minus with spin-label. The spectra at the right are simulations, and the spectra at the left are the observed spectra. The binding site occupancies with spin-label are (a) 100%, (b) 20.03%, (c) 4.96%, and (d) 2.57%.

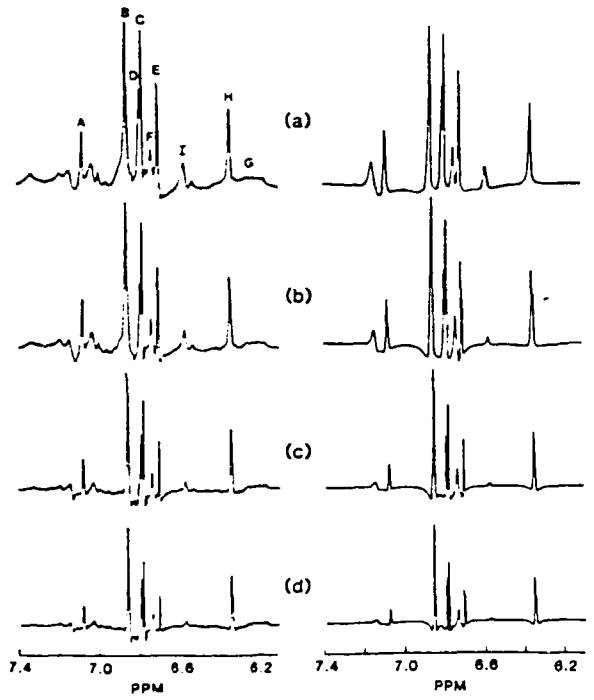

FIGURE 2: Difference spectra of with DNP minus with spin-label. The spectra at the right are simulations, and the spectra at the left are the observed spectra. The binding site occupancies with spin-label are (a) 100%, (b) 25.2%, (c) 7.52%, and (d) 3.9%.

Figure 1 shows the simulated and observed spectra for several of the difference spectra in the without minus with spin-label titration. All spectra are to the same scale. Figure 2 shows results for the with DNP minus with spin-label titration. The spectra in Figures 1 and 2 are to different scales. Only four of the six recorded spectra in each data set are shown. The spectra for the third and fifth highest occupancy are omitted in each case.

The changes in chemical shift that occur on binding can be observed by comparing Figure 1a and Figure 2a. These changes make the amplitudes of pairs of lines interdependent in several cases. The lines labeled C and D are very close to each other, and the negative signals from each of these protons

Table I: Spectral Parameters for without minus with Spin-Label Titration

proton	chemical shift[a] (ppm)	line width[a] (Hz)
A	7.126	9.4
B1	6.864	6.9
B2	6.857	7.7
C	6.810	5.1
D	6.799	8.4
E	6.722	4.8
F	6.693	12.1
G	6.487	10.8
H	6.435	10.2
I	6.595	17.0

[a] The chemical shifts and line widths individually do not have any significance beyond ±0.01 ppm and ±1 Hz. The shapes of overlapping signals are more sensitive to the relative values of line positions and widths than these limits, as indicated by the number of significant figures used in the table.

Table II: Spectral Parameters for with DNP minus with Spin-Label Titration

proton	chemical shift[a] (ppm)	line width[a] (Hz)
A	7.082	5.8
B1	6.865	6.0
B2	6.865	2.9
C	6.784	2.8
D	6.795	4.2
E	6.706	3.3
F	6.738	8.25
G	6.135	br[b]
H	6.349	5.15
I	6.575	11.0

[a] The chemical shifts and line widths individually do not have any significance beyond ±0.01 ppm and ±1 Hz. The shapes of overlapping signals are more sensitive to the relative values of line positions and widths than these limits, as indicated by the number of significant figures used in the table. [b] Broad.

affect the other's amplitude in one or both of the titrations. The negative feature from line F moves through the position of the positive signal from line E in the without minus with spin-label titration, thus affecting its amplitude. The fitting algorithm allows for these interdependencies. It is more difficult to model the small signal at slightly higher chemical shift than line A and its effect on the observed amplitude of line A. This is probably not a tyrosine signal (or even necessarily from a single source). Both histidine and unexchanged peptide amide protons would show signals in this spectral region. Since the number of protons giving rise to this signal cannot be determined, it cannot be scaled properly with respect to the tyrosine signals in the calculation.

Table I lists spectral parameters derived from the without spin-label minus with spin-label spectra. The chemical shits of the protein tyrosine resonances and the line widths derived from the best fit to the titration data are shown in the table. Table II shows results for the with DNP minus with spin-label difference spectra. Identical labels for the protons in the two tables indicated that the protons are from the same amino acid. The method used to interrelate signals in the two spectra has been discussed previous (Anglister et al., 1984). Note that the line widths of a given proton are not the same in the two types of spectra and are almost always narrower in the DNP complex. Examination of the spectra in Figures 1 and 2 shows that the line shapes calculated are in most cases in good agreement with the observed spectra, so that this difference in calculated line width is not likely to be an artifact of the different scaling of the two titrations.

This narrowing of line width in the with DNP spectrum is interesting. If some paramagnetic impurity is less able to penetrate the protein when hapten is bound, a narrowing effect

Table III: Results with Relevance to Model Building

proton	change in chemical shift (without spin-label to with DNP in ppm)	distance[a] (Å)	chain
A	0.044	12.9	H
B1	−0.001	12.3	H
B2	−0.001	<7	H
C	0.026	11.5	L
D	0.004	9.0	L
E	0.016	13.7	H
F	−0.045	10.85	L
G	0.352	ND[b]	L
H	0.086	<7	H
I	0.020	16.0	L

[a] The calculated distances are derived from averages of the inverse sixth power of the electron spin–proton spin distance and may represent averages over different molecular conformations. [b] Not determined.

might be observed. We have accumulated spectra in the presence and absence of dissolved oxygen and see no difference in line width, ruling out the most likely candidate for the broadening agent. A second possibility is that there is more mobility for tyrosine residues not in contact with the hapten when hapten is bound. We have previously stated that the large number of resonances showing some change in chemical shift might indicate a significant change in average conformation. On the other hand, there is clearly an immobilizing effect on the tyrosine giving rise to resonance G, along with the very large change in chemical shift seen for this resonance.

Table III shows the change in chemical shift undergone by each Fab tyrosine resonance upon DNP binding. This table also shows the calculated distance of each of these protons from the paramagnetic center of the hapten. Distances of less than 7 Å cannot be resolved because of the magnitude of the off rate of the hapten (Anglister et al., 1984), and are all labeled as <7. The change in chemical shift and the distance from the spin are the two parameters with strongest implications for structural models of the antibody. An L or H is included in the last column to indicate a light- or heavy-chain origin for the resonance (Anglister et al., 1985). The distances given here are in agreement with those reported previously (Anglister et al., 1984), except for line D. The overlap in line D with line C is likely to be a source of error in the previous calculations.

The value for the distance of line G from the paramagnetic center could not be determined. This is due to the large change in chemical shift undergone by this signal during the without minus with spin-label titration. The negative signal from the partially occupied spectrum moves out from under the positive signal at very low occupancy and is exchange broadened independent of any paramagnetic effect. A distance value for protons giving rise to signal G cannot be obtained from the with DNP minus with spin-label titration due to the extremely poor signal to noise for this line caused by its large line width.

Discussion

It can be seen from the simulated spectra (Figures 1 and 2) that the computer analysis used in this work gives a good approximation to the observed spectra. The simulation of the spectra allows the analysis of overlapping signals. The rate of appearance of a resonance in a titration is dependent on the line width, but line-width measurements are not very accurate when determined directly from the spectra. Spectral simulation forces the line widths to be mutually consistent. The derived line widths also provide a quantitative confirmation of the qualitative observation that resonances in the DNP complex are narrower than in the free Fab, as discussed under Results.

The spectra discussed here yield results with structural implications. We have previously discussed a computer model of the antibody and used it to assign likely tryptophan residues to specific resonances (Anglister et al., 1987). The assignment of tyrosine resonances is not so straightforward. There are two heavy-chain tyrosines (33 and 50) which are candidates for signals B and H, but the large number of tyrosine residues makes this assignment very provisional. Further complicating the situation is the lack of an obvious candidate for resonance G. This is the tyrosine resonance that undergoes a large change in chemical shift upon hapten binding. One reason for the lack of a candidate is that the model was computed for an empty binding site, and the change in chemical shift could be due to conformational change in the Fab. There are at least four light-chain tryosine residues that could be candidates under these assumptions, including the three tyrosines in the second hypervariable loop of the light chain.

The type of analysis described here is being applied to titrations done with several different deuteriations. It is hoped that a collection of these measurements will lead to an understanding of the solution structure of this antibody and, in conjunction with the crystallographic and mutagenesis studies under way, will lead to an improved understanding of antibody–hapten binding in terms of both structure and kinetics.

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Appendix

Magnetization Transfer Due to Chemical Exchange. The chemical components of the system are Fab, spin-label (SL), DNP-diglycine (DNP), and the complexes of Fab with spin-label (FabSL) and of Fab with DNP (FabDNP). Two reversible reactions are assumed:

$$\text{FabSL} \rightleftharpoons \text{Fab} + \text{SL} \quad (1)$$

$$\text{FabDNP} \rightleftharpoons \text{Fab} + \text{DNP} \quad (2)$$

Thus, we assume that FabSL can only be converted to FabDNP through the intermediate free Fab.

Let M_S be the macroscopic magnetization association with a specific proton in the Fab fragment when spin-label is bound and M_D be the magnetization of the same proton when DNP is bound. Let M_0 be the magnetization of this proton when the binding site is empty. The changes of the macroscopic magnetization due to chemical exchange can then be written as follows:

$$\dot{M}_S = -k_{S0}M_S + k_{0S}M_0 \quad (3)$$

$$\dot{M}_D = -k_{D0}M_D + k_{0D}M_0 \quad (4)$$

$$\dot{M}_0 = -k_{0S}M_0 - k_{0D}M_0 + k_{S0}M_S + k_{D0}M_D \quad (5)$$

where k_{S0} is the first-order rate constant for dissociation of the Fab–spin-label complex and k_{0S} is the pseudo-first-order rate constant for reassociation.

Assume experimental conditions where there is always an excess of spin-label and/or DNP such that the binding sites of the Fab fragments are almost always occupied. Under these circumstances, the concentration of free Fab is very small, and we may set $\dot{M}_0 = 0$. We then obtain the following expression for M_0:

$$M_0 = \frac{k_{S0}M_S + k_{D0}M_D}{k_{0S} + k_{0D}} \quad (6)$$

Substitution of this expression for M_0 in eq 3 and 4 allows these equations to be rewritten as

$$\dot{M}_S = -k_{SD}M_S + k_{DS}M_D \quad (7)$$

$$\dot{M}_D = -k_{DS}M_D + k_{SD}M_S \quad (8)$$

where

$$k_{SD} = \frac{k_{S0}k_{0D}}{k_{0S} + k_{0D}} \quad (9)$$

$$k_{DS} = \frac{k_{D0}k_{0S}}{k_{0S} + k_{0D}} \quad (10)$$

Thus, the equations describing the change of magnetization due to chemical exchange have the form of a two-site exchange reaction, as though the reaction

$$\text{FabSL} + \text{DNP} \rightleftharpoons \text{FabDNP} + \text{SL} \quad (11)$$

took place with rate constants k_{SD} and k_{DS}.

In the form of the Bloch equations described in Wien et al. (1972), the parameter τ is used, and for the present problem

$$\tau = 1/k_{SD} + 1/k_{DS} \quad (12)$$

In order to put this equation into useful form, we can express the time τ in terms of the true first-order off rate constants k_{S0} and k_{D0} and the fractional occupation of the binding site by spin-label and DNP, namely, f_S and f_D. To do this, first note that

$$k_{0S} = k'_{0S}[\text{SL}] \quad (13)$$

$$k_{0D} = k'_{0D}[\text{DNP}] \quad (14)$$

where k'_{0S} and k'_{0D} are the true second-order rate constants for the reverse reactions in eq 1 and 2. The equilibrium constant for reaction 1 is

$$K_S = \frac{[\text{Fab}][\text{SL}]}{[\text{FabSL}]} = \frac{k_{S0}}{k'_{0S}} \quad (15)$$

and for reaction 2

$$K_D = \frac{[\text{Fab}][\text{DNP}]}{[\text{FabDNP}]} = \frac{k_{D0}}{k'_{0D}} \quad (16)$$

By combining eq 15 and 16 with eq 12, we obtain the following expression:

$$\tau = \frac{1}{f_D k_{D0}} + \frac{1}{k_{S0} f_S} \quad (17)$$

or

$$\tau = \tau_{D0}/f_D + \tau_{S0}/f_S \quad (18)$$

where τ_{D0} and τ_{S0} are the lifetimes for the two haptens in the combining site. (Note that for the two-site exchange case, where there is no DNP-diglycine, $\tau = 1/k_{S0}f_S$. The effect of the DNP-diglycine is effectively to slow down the rate of entry of the spin-label into the binding site.)

Registry No. DNP-glycine, 1084-76-0.

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